Proc. Nat. Acad. Sci. USA
Vol. 70, No. 12, Part I, pp. 3478–3482, December 1973

Early Differentiation of Glucagon-Producing Cells in Embryonic Pancreas: A Possible Developmental Role for Glucagon*

(electron microscopy/embryogenesis/endocrine cells/hormone)

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Communicated by Clifford Grobstein, July 16, 1973

ABSTRACT **Glucagon and insulin are first detectable at the onset of rat pancreas organogenesis. Initially, the specific activity of glucagon is approximately 100-fold higher than that of insulin. At this early stage, endocrine storage granules, similar to α granules, are identifiable in electron micrographs. The granule characteristics, as well as the relative hormone levels, suggest that the early population of differentiated endocrine cells is in fact composed of glucagon-producing (A) cells. This high level of glucagon is present in the embryo much earlier than the metabolic processes thought to be controlled by this hormone. Moreover, glucagon-producing cells may be the first endocrine cells to differentiate. Other known endocrine products accumulate later, during the terminal stages of organogenesis. These observations suggest that glucagon may have a regulatory function in early embryogenesis.**

The islets of the differentiated rat pancreas contain A and B cells, which are responsible for the production of glucagon and insulin, respectively, and D cells, whose function has yet to be defined. Until recently, the endocrine cells have been assumed to differentiate well after the appearance of the pancreatic diverticulum (3–5). However, Clark and Rutter (6), using a sensitive immunoassay, detected low levels of insulin at the time of the initial formation of the pancreatic diverticulum. Moreover, Wessells and Evans (7) demonstrated that granulated (differentiated) endocrine cells were present at this time. It was assumed, therefore, that these differentiated cells were B cells.

The present studies show that glucagon is present at high levels at this early stage of development and that the granule-containing cells are instead A cells. Endocrine B cells, like acinar cells, appear later. It is possible that the A cells are the first endocrine cells to differentiate in the embryo and that glucagon, therefore, is the first hormone produced at functional levels.

MATERIALS AND METHODS

Collection and Preparation of Tissues for Assay. Pregnant Sprague–Dawley rats dated according to the vaginal plug procedure (8) were decapitated, and the embryos of an appropriate gestational age were transferred to dissecting dishes containing Earle's balanced salt solution and 1.0% bovine-serum albumin. Pancreatic rudiments, excluding most of the associated mesenchyme, were excised and placed in polyethylene tubes (microfuge tubes, Spinco) on dry ice. The samples were stored at −70° until the time of assay, when they were thawed and subsequently sonicated in an aliquot of glass-distilled water. The crude homogenates were immediately monitored for hormone activity and protein content. Routinely, three dilutions were made of each sample, and each dilution was assayed in duplicate.

Insulin Assay. Insulin was assayed according to a micromodification of the double-antibody technique of Morgan and Lazarow (9). Previously (6) beef insulin was used as a standard, while our more recent studies use the homologous rat insulin and a different combination of reagents to enhance the precision and sensitivity of the assay. In the current procedure, dilutions of reagents, standards, and samples are made with 0.5% crystalline bovine-serum albumin (Miles Laboratories) in 0.05 M phosphate (pH 7.0). The assay is carried out in three successive stages on each sample:

I (4°, 18–24 hr): 30 μl of unknown sample or rat insulin standard (Novo Research Institute) containing 10–80 pg of insulin and 20 μl of anti-ox insulin serum prepared in guinea pig (Burroughs Wellcome), diluted 1:20,000.

II (4°, 24 hr): 100 pg of ^{125}I beef insulin (Amersham-Searle).

III (4°, 18–24 hr): 20 μl of normal guinea pig serum diluted 1:40 and 50 μl of anti-guinea pig serum prepared in rabbit (Miles Laboratories) diluted 1:1.5.

After the third incubation, the precipitates are collected by centrifugation (4°, 3000 rpm), washed, and counted. The ratio of the labeled hormone bound in the presence and absence of standard rat insulin is conveniently plotted by the logit transformation (10). The displacement of radioactive insulin by the "insulin-like" material in the sample is then compared with the standard curve. The results are not affected by the inclusion of a proteolytic inhibitor (pancreatic trypsin inhibitor, Kunitz, 1 μg/10 μg of pancreatic protein).

Glucagon Assay. Glucagon was determined by a double-antibody immunoassay using antiglucagon serum (prepared in guinea pig) diluted 1:300 and anti-guinea pig serum (prepared in rabbit). Both antisera were prepared by Dr. James Vance in the laboratory of Dr. R. H. Williams. The procedure, which has been described for the analysis of serum preparations (11), was tested for its applicability to crude pancreatic sonicates. The suggested level of pancreatic trypsin inhibitor (Trasylol, 1000 Kallikrein Inactivator Units, FBA Pharmaceuticals) prevents the proteolytic degradation of glucagon during the assay. Furthermore, added glucagon was quantitatively recovered from such pancreatic homogenates.

* A preliminary account of this study was presented at the American Diabetes Association Meeting, 1969 (1, 2).

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The possibility that crude homogenates may nonspecifically interact with the antibody system was tested by measuring the crossreactivity of increasing concentrations of an *Escherichia coli* sonicate. Protein concentrations greater than 100 μg per assay tube produced a low, yet significant, displacement of labeled hormone (insulin or glucagon). However, the lowest specific activities of glucagon determined required no more than 20 μg of total pancreatic protein; the lowest levels of insulin determined required less than 10 μg of total protein.

The behavior of glucagon in the immunoassay is defined by appropriate concentrations of beef-pork glucagon standard. The glucagon activities then measured is the amount of rat glucagon immunologically equivalent to beef-pork glucagon. However, it has recently been shown that the amino-acid compositions of beef, pork, and rat glucagon are identical (12); thus, in this assay procedure identical molecules compete for the glucagon antibody.

Electron Microscopy. Pancreatic rudiments, freshly dissected or cultured organotypically, were fixed in 2% distilled glutaraldehyde in 0.44 M phosphate buffer (pH 7.4). After a minimum of 60 min, the samples were briefly washed and fixed in 0.15 M osmium tetroxide in the same buffer. Tissues were dehydrated and embedded according to Luft (13). Thin sections were cut with a diamond knife and harvested on 100 or 75 mesh grids, or on L2 × 1 grids for topographical work. To enhance contrast, the sections were treated with lead acetate, sometimes preceded by uranyl acetate, and examined in an RCA EMU 2A, 2C, or 3G electron microscope.

RESULTS

Analysis of Hormone Levels. The pattern of accumulation of insulin and glucagon immunoreactivity is presented in Fig. 1. Insulin is first assayable during the period of the initial formation of the pancreatic diverticulum (11th day of gestation, 20–25 somites). The concentration of insulin (normalized to total protein content) increases from an undetectable level at day $10^1/_2$ to a steady-state level which begins at day 12 and persists through the 14th day. A second dramatic increase in specific concentration occurs between days 14 and 20. This biphasic profile for the accumulation of insulin contrasts with that observed for glucagon. Glucagon is also present with the appearance of the pancreatic diverticulum (20–25 somites, 11th day of development), but at levels 100-fold higher than that of insulin. The glucagon specific activity decreases by about a factor of 5 during subsequent development, reflecting a 5- to 10-fold increase in the protein content of the exocrine cells, the predominant cell in the tissue. Thus, the glucagon content expressed on a per cell basis remains essentially constant during development. After the secondary rise in insulin content, the ratio of this hormone to glucagon approaches 25, reflecting the predominance of B over A cells in the late embryonic pancreas.

The reliability of our observations, especially during the early developmental stages, depends upon the specificity and the increased sensitivity of the immunoassays used. For example, the plateau level of insulin on days 12–14, if relatively low, exceeds the limiting sensitivity of the assay by at least a factor of 10. Glucagon levels exceed the sensitivity of the immunoassay by at least 40-fold. In addition, several tests were performed to confirm the specificity of the immunoassays. The insulin assay detects insulin, proinsulin, and related intermediates (16). Thus, changes in the ratio of proinsulin to insulin during development would not be detected by this procedure. Other studies suggest, however, that there is no major change in the relative amount of proinsulin during the course of development (Rall, L. & Rutter, W. J., unpublished observations). For glucagon, an immunologically active precursor molecule has not been ruled out (17). However, neither secretin, which is quite similar to glucagon in structure (18), nor rat insulin crossreact in the glucagon assay. It is known that some antisera prepared against glucagon crossreact with a molecule found in the gut (19). However, we have obtained similar results using an antibody specific for pancreatic glucagon (provided by Dr. R. H. Unger, University of Texas, Southwestern Medical School, Dallas, Tex.). The specificity of the immunoassay is further indicated by demonstrating that the two hormones are found uniquely in the pancreas of the embryo. Insulin, within the limits of the assay, was undetectable in other embryonic organs (liver, spleen, muscle, heart, or kidney). Similarly, glucagon immunoreactivity was not detected in crude homogenates of day 14 intestinal sections.

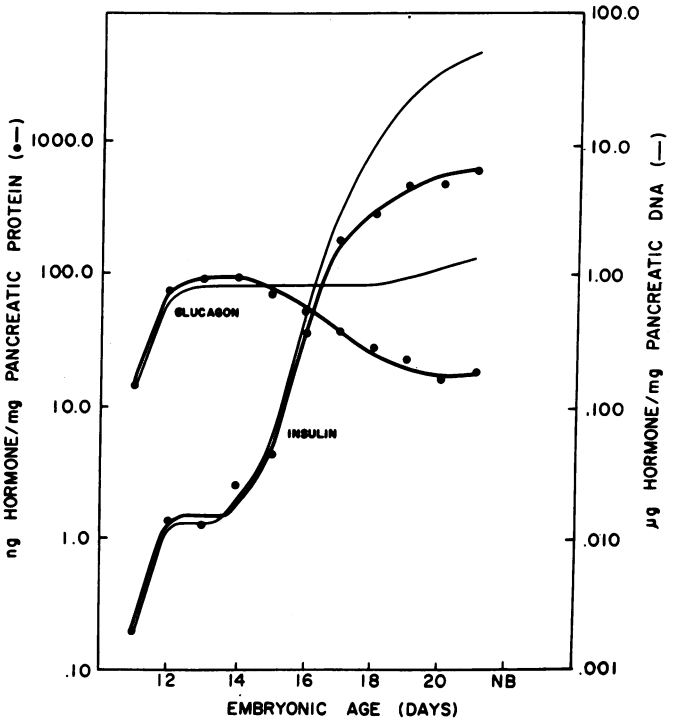
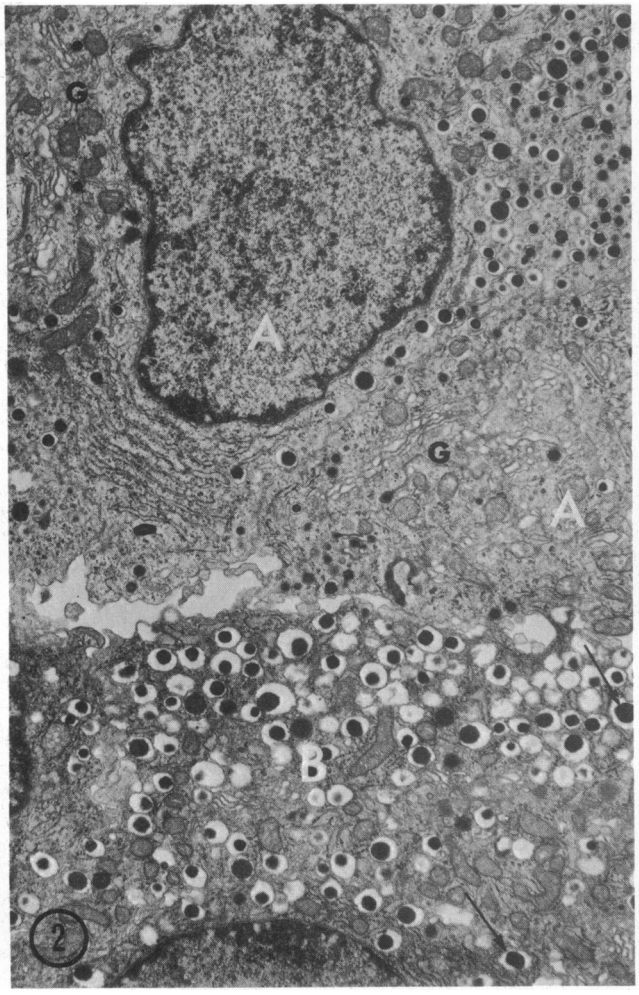


FIG. 1. Developmental profiles of the endocrine rat pancreas. Rudiments were dissected from embryos of the indicated age (*NB*, newborn), pooled, sonicated, and assayed for insulin by a micromodification of the double-antibody immunoassay of Morgan and Lazarow (9). The assay used for glucagon is based on a double-antibody immunoassay developed in the laboratory of Dr. R. H. Williams (11). Protein determinations were made by a micromodification of the Folin–Lowry protein assay (14). DNA levels were determined by the fluorometric procedure of Kissane and Robbins (15).

The shape of the insulin developmental profile remains the same whether beef, as previously reported (6), or rat insulin (this publication) is used as a standard in the immunoassay. However, we now find that the absolute level of insulin measured during a particular developmental stage is significantly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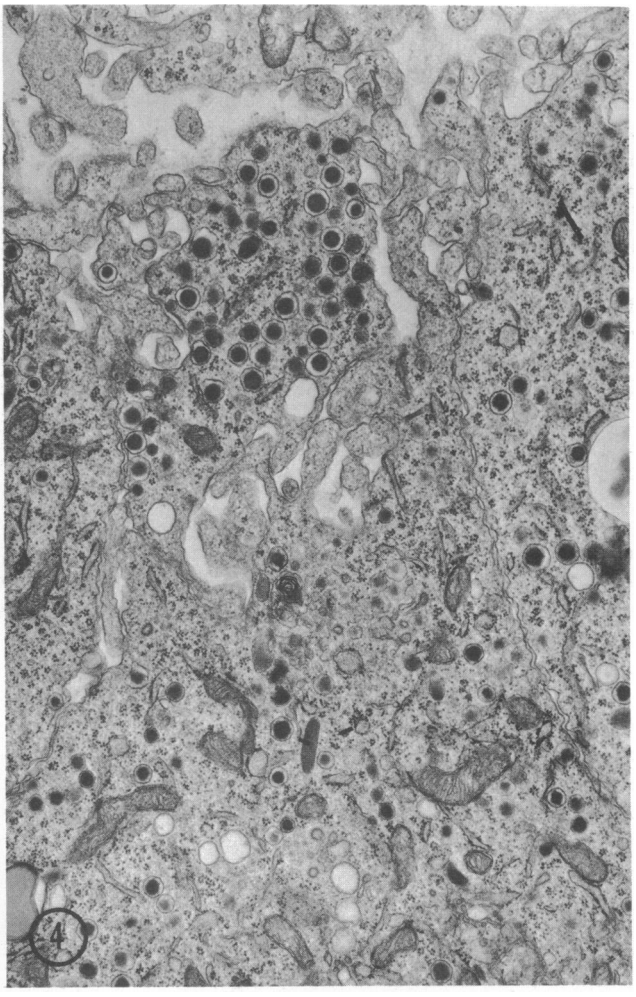
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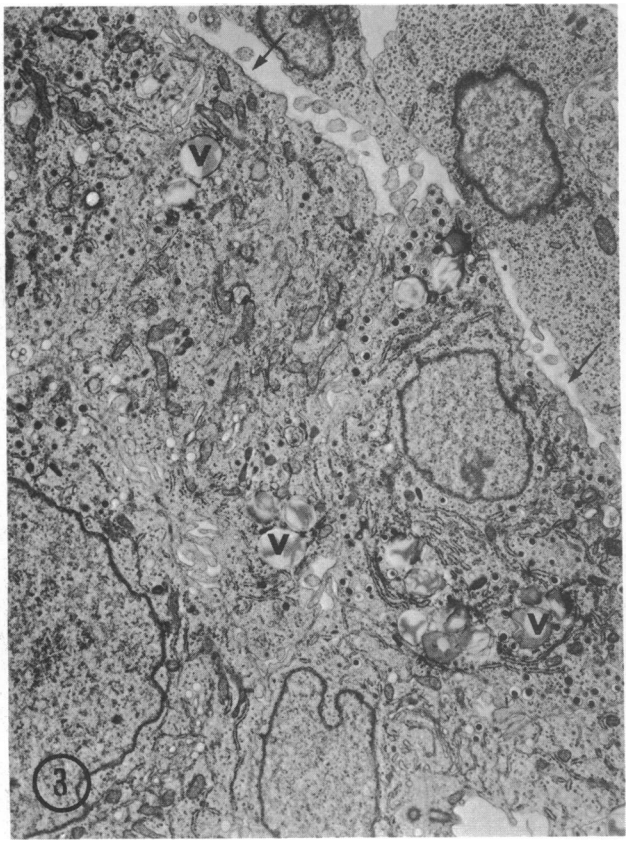
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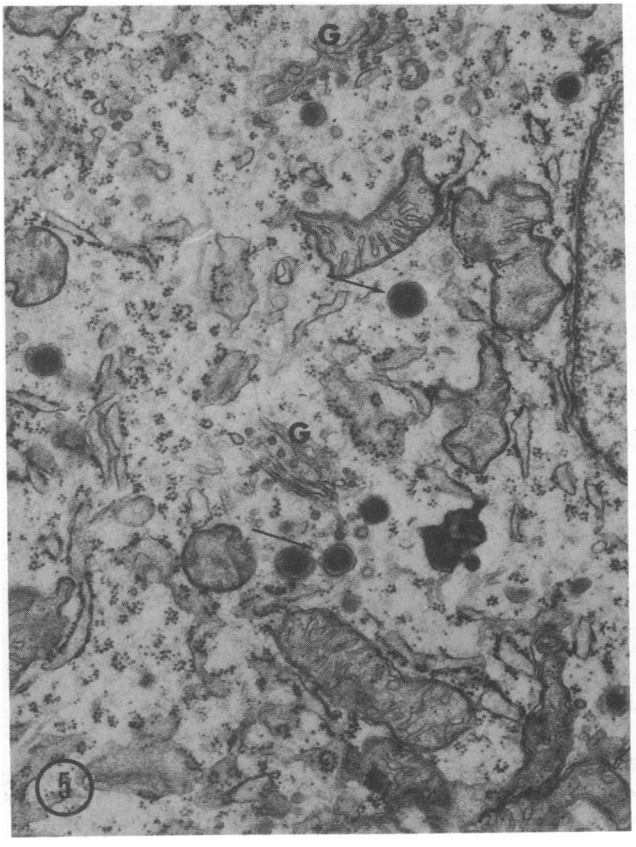
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higher. This discrepancy in the two assays is probably related to the heterogeneity in the sample and standard. For example, the assay levels of Clark and Rutter (6) in the late embryonic pancreas agree well with earlier studies using beef insulin standards (20), whereas the insulin content of the late embryonic pancreas found in the present study agrees with those using homologous rat insulin as a standard (21, 22).

The interpretations of previous studies describing pancreatic endocrine development have been limited by the assay procedures available. Murrell *et al.* (20), using a less sensitive immunoassay, measured insulin levels during the late embryonic stages and by a linear extrapolation predicted the initiation of insulin synthesis between days 16 and 17. Grillo (23), in contrast, using an immunofluorescence method, reported the presence of insulin earlier in development, just at the beginning of the dramatic increase in specific activity. Grillo *et al.* (24), as well, measured the level of glucagon-like material based on the activation of glycogen phosphorylase in rabbit-liver slices and reported its presence as early as the 14th day. However, it was noted that up to the 19th day of gestation, the activity of fetal pancreas was not greater than than of spleen, liver, or the gastrointestinal tract of newborn or adult rats, an observation that is not in agreement with our assays. However, since it is now known that the activation of glycogen phosphorylase in crude tissue preparations is affected by a number of factors, this assay can no longer be considered specific for glucagon.

Ultrastructural Analysis. In rats, there are four ultrastructural parameters for distinguishing A from B cells: (*1*) the distance separating the granule from its membrane, (*2*) the density of the granule, (*3*) the shape of the granule, and (*4*) the size of the Golgi complex. Thus, α granules are uniformly dense and round and the distance separating the granule from its membrane is small. In contrast, the density of β granules varies from one granule to another and they are generally less dense than α granules. There is a relatively large space between the β granule and its membrane, and a significant proportion of the granules are crystalloid. Finally, the Golgi complex in A cells is smaller and more spherical than in B cells.

The endocrine cell types are readily differentiated when they are together in a single section, as in Fig. 2. In such sections of 17- to 22-day fetal pancreases, one observes A, B, and D cells. During this period, B cells predominate, A cells are frequently seen, and D cells are rare. Before day 15, however, there is only one population of endocrine cells and the secretory granules resemble those of A cells rather than B cells. Fig. 3 is a low-field magnification of a group of endocrine cells from the upper surface of the dorsal pancreatic epithelium, 35 somites, 12th day of development. Each cell is of the same type and, as shown in Fig. 4, the ultrastructural features of these cells are comparable to the A cells seen in Fig. 2. The granules are uniformly dense, regular in shape, and in close proximity with their membrane. These secretory granules first appear with the initial formation of the pancreatic diverticulum, 20–25 somites, 11th day of development.

As shown in Fig. 5, there are only a few granules appearing in a given section. Yet, they are characteristically of the α type, and in the next few days of development (days 12–15) both the number of this cell type and the number of granules per cell increase. Thus, it is possible to convincingly argue that the earliest differentiated cells in the pancreas are in fact A cells. Moreover, granulated B cells first appear only after the 15th day of development. Subsequently, there is a rapid augmentation of B cells and a dramatic inversion in the ratio of B to A cells in the endocrine population. This observed late accrual of cells containing β granules is supported by earlier histological studies. Most authors agree that B cells are first sensitive to aldehyde fuchsin staining at about day 18 in rats even though islets are present earlier in development (4, 23). Munger (25), using ultrastructural techniques, described endocrine cells containing granules at days 13–15 in mice, corresponding to days $14^1/_2$–16 in the rat. The secretory granules had the characteristics of α granules, but on the basis of information then at hand, Munger believed they were more likely to be B cells and therefore termed these cells "immature B cells." More recently, Wessells and Evans (7) described endocrine cells containing secretory granules at the time of the appearance of the mouse pancreatic diverticulum. Since our laboratory had reported low levels of insulin during this stage (26) and because of the presumed late differentiation of A cells (4), Wessells and Evans attributed these granules to B cells. However, our current results suggest that these early granules are in fact α granules, as in rat embryos.

This sequential development of the endocrine A and B cells also explains a previously perplexing observation. The early cells containing endocrine granules are resistant to the β-cytotoxic action of streptozotocin. Susceptibility to the compound is coincident with the appearance of differentiated B cells (27).

DISCUSSION

This coordinated biochemical-morphological study defines the developmental pattern for the endocrine A and B cells and their specific products. Our experimental observations, especially during the early developmental stages, are a result of more sensitive assay methods and more refined ultrastructural techniques. Both insulin and glucagon are detectable during

FIG. 2. Fetal rat pancreas, day 20 and 12 hr in culture. As in adults, both endocrine A and B cells are present. The distinction is made primarily by considering the distance separating the granule from its membrane. Note the dense granules in the Golgi region of the A cells and the two crystalloid granules in a B cell (*arrow*). *G*, Golgi complex; *A*, A cell; *B*, B cell. ×10,000.

FIG. 3. Embryonic pancreas, 35 somites, a group of endocrine cells from the top surface of the dorsal pancreatic epithelium. These early islets contain only one type of endocrine cell, and the granulation is of the α type. The pancreatic epithelial tissue is separated from the mesenchyme by a continuous basal lamina (*arrows*). The function of the vesicles (*v*), which frequently appear in early endocrine cells, is not known. ×6,000.

FIG. 4. Embryonic pancreas, 35 somites (12th day of development). In comparison with Fig. 2, these cells are structurally analogous to A cells. ×16,000.

FIG. 5. Embryonic pancreas, 24 somites (11th day of gestation), onset of rat pancreas organogenesis. Differentiated (granule-containing) endocrine cells and the initial pancreatic bulge appear simultaneously. The proximity of the granule and its membrane and the uniform density of the granules are characteristic of α granules (*arrows*). *G*, Golgi complex. ×18,000.

the initial formation of the rat pancreatic diverticulum. However, the absolute levels and developmental profiles of the two hormones are dramatically different. The pattern of accumulation of insulin is biphasic; an initial low level of insulin is maintained from the 12th to the 14th day and subsequently, a several hundred-fold increase in specific activity ensues. The digestive proteins in pancreatic exocrine cells accumulate in a similar step-wise fashion (26–28). In contrast, at the time of the formation of the pancreatic diverticulum glucagon is already present at high levels that are maintained during subsequent development. This level of glucagon is similar to that found in the adult pancreas (22). Furthermore, there is only one population of cells containing secretory granules during the early stages (days 11–15) of development and the ultrastructural features are strongly suggestive of A cells. The increase in both the secretory granules within individual cells and the number of granulated cells between 24 somites (11th day) and 40 somites (12th day) is consistent with the increase in glucagon and not with the stable and low level of insulin found between days 12 and 14. In addition, the subsequent appearance of typical B cells correlates well with the increase in immunoassayable insulin, just like the appearance of zymogen granules coincides with the rapid accumulation of specific enymes in exocrine cells (26, 28). Therefore, we conclude that these early granule-containing cells are A cells and that in the embryonic rat pancreas there is a sequential differentiation of endocrine A and then B and acinar cells.

Furthermore, glucagon may be the first hormone synthesized at functional levels in the embryo. As far as is known, the pituitary, the thyroid, and the adrenal glands all differentiate and produce functional levels of their respective hormones considerably later than glucagon (29–33). We have not yet demonstrated that glucagon is secreted at these early times; but the competence for insulin secretion coincides with the appearance of secretory granules (34). Therefore, it is reasonable to believe that the early A cells are competent to secrete glucagon.

What role could glucagon play in the early embryo? The principal sites of glucagon action are currently thought to be the regulation of glucose (glycogen) metabolism in the liver, the mobilization of lipids, and the regulation of insulin secretion. None of these functions can be implicated at the time of A-cell differentiation. High levels of glucagon exist 6–7 days before glycogen accumulation in the liver (35) and 10 days before lipid droplet formation in adipose tissue (36). Furthermore, B cells do not differentiate (form β-secretory granules) until several days later (27, 28). Glucagon probably exerts its various effects by increasing the levels of cyclic AMP, which in turn may lead to a decreased rate of cell proliferation and to changes in macromolecular synthesis (37). A general role of glucagon in the regulation of growth and differentiation in embryonic cells, therefore, seems plausible.

This study was supported by the USPHS Grants HD-04617, AM 02456, AM 05020, and AM 12137, NSF Grant 3-5256, The United Health Fund, and NSF GB 14896. R.L.P. was a recipient of a Research and Development Award of the American Diabetes Assoc. (1970–1972).

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